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Hard ticks circulate *Anaplasma spp.* in South-Khorasan province, Iran

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ABSTRACT

Ticks are vectors for several important zoonoses including different species of *Anaplasma*. The present study aims to determine the presence of *Anaplasma spp.* in hard ticks collected from livestock of South-Khorasan province, Iran. A total of 684 livestock were sampled and 269 ticks were collected. Two genera and 6 species of ticks were identified including *Rhipicephalus sanguineus, Hyalomma detritium, Hyalomma marginatum, Hyalomma anatolicum, Hyalomma asiaticum, Hyalomma dromedarii* and *Hyalomma spp.* Eleven *Hyalomma* nymphs and 3 *Rhipicephalus* nymphs were also identified. 100 Out of 269 ticks were chosen for molecular detection. DNA was extracted followed by PCR technique to detect *Anaplasma spp.* The presence of *Anaplasma spp.* was confirmed in 20 out of 100 tested samples (20%). All positive samples collected from Birjand county were *Rhipicephalus sanguineus*. Results of the present study showed a relatively high infection rate of *Anaplasma* in hard ticks in South-Khorasan Province.

Keywords

Ixodidae, PCR, Hyalomma, Rhipicephalus

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Abbreviations

PCR: Polymerase chain reaction
BLAST: Basic local alignment tool
MSP-4: Major surface protein 4

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Anaplasmosis refers to a disease of animals and humans, caused by obligate intra-erythrocytic bacteria (Family *Anaplasmataceae*: order Rickettsiales), which poses significant livestock economic constraints to countries due to reduction in milk and body weight, abortions, veterinary expenses, and finally animal losses [1]. Species of veterinary interest include *A. bovis, A. centrale, A. marginale, A. ovis, A. phagocytophilum* (common species between humans and animals), and *A. platys.* Clinical evidence of anaplasmosis in livestock is characterized by an acute onset of fever, weight loss, pale or yellowish mucosa, anorexia, milk reduction, and death (if not treated appropriately). After recovery from infection, the animal remains a source of infection forever [2].

Ticks are vectors for several important zoonoses including spotted fever, Rocky Mountains fever, Siberia tick typhus, tularemia, Lyme disease, tick-borne relapsing fever (TBRF), Crimean-Congo hemorrhagic fever (CCHF), babesiosis, and anaplasmosis. Alongside pathogen transmission, tick-induced direct losses to livestock such as bite stress, production loss, physical damage, anemia, and poisoning are also considerable [3]. Ticks are categorized into three families: *Ixodidae, Argasidae*, and *Nuttalliellidae*. The *Ixodidae* family, also called hard ticks, contains most ticks of veterinary importance [4].

According to statistics published by the statistics center of Iran, the population of large and small ruminants in South-Khorasan province is about 140,000 and 34,807, respectively, which play an important role in the economy and life of the people of the region [5]. On the other hand, South Khorasan province shares a 460 km border with Afghanistan which is endemic for many diseases including malaria, leishmaniasis, tick-borne encephalitis, Crimean-Congo hemorrhagic fever, and anaplasmosis. The above facts imply the importance of epidemiological studies on vectors and ruminants in this area [6]. The present study aims to determine the presence of Anaplasma spp. in the hard ticks, collected from livestock in South-Khorasan province, Iran.

Study area and sample collection

Birjand, Qaen, Khusf, Darmian and Sarbisheh counties from South Khorasan province (32.8653°N 59.2164°E) were surveyed. A total of 684 livestock (sheep, goats, cows and camels) were sampled in summer 2019. Multistage random sampling method was used for the collection of tick samples. Hard ticks were randomly collected using forceps and then were placed into labeled tubes. All samples were transferred on ice to the Department of Medical Entomology, School of Public Health, Tehran University of Medical Sciences, Iran for species identification. Ticks were identified at the level of species under stereomicroscope according to valid morphological keys [7].

Molecular detection of Anaplasma

100 out of 269 ticks were chosen for molecular detection according to sex, collection area and tick life stage. Ticks were placed into 70% ethanol for 15 min; air dried and kept in separate tubes. Incubation in liquid nitrogen for 5 min followed by grinding was performed for each tick. DNA extraction was performed by the Exgene extraction kit (GeneAll®, Korea) according to the manufacturer's guidelines. 16s ribosomal RNA gene was targeted to amplify a 524 bp fragment by nested-PCR using two primer sets as follows: Ehr1 5′-GAACGAACGCTGGCGGCAAGC-3′ and Ehr2 5′-AGTA[T/C]CG[A/G]ACCAGATAGCCGC-3′ for the first step and Ehr3 5′-TGCATAGGAATCTACCTAGTAG-3′ and Ehr4 5′-CTAGGAATTCCGCTATCCTCT-3′ for the second step of nested-PCR. Also, an amplicon of 464-bp msp4 was amplified using primers Fmsp4: 5'-GTYARRGGCTAYGRCAAGAG-3' and Rmsp4: 5'-AGTRAACTGGTAGCTWATYCCA-3'. The procedure of nested-PCR was similar to Rar et al. with some modifications in the PCR thermal program [8,9]. 25 µL reaction mixtures contained 2.5 µL PCR buffer, Tris-HCl (10×) (pH 9.0), 0.75 µL MgCl2, 0.5 µL dNTPs, 0.2 µL Taq DNA polymerase, 1 µL of each of forward and reverse primers, and 2 µL of extracted DNA [9]. Confirmed positive DNA samples for *Anaplasma* species were obtained from Department of Parasitology, Faculty of Veterinary Medicine, University of Tehran, Iran. Double-distilled water was used as the negative control. Products were subsequently analyzed by gel electrophoresis.

Sequencing and phylogenetic Analysis

A pool of 10 positive ticks were sent to Codon Genetic Group® (Tehran, Iran) for sequencing. Sequences were then checked to correct any sources of error using the Chromas® software. The sequence were then blasted by BLAST, National Institute of Health, USA (http://www.ncbi.nlm.nih.gov/BLAST) and submitted to GenBank with accession number [MW428433].

The present sequence was aligned using MEGA7 software (CLUSTALW algorithm) and used in phylogenetic tree construction. Maximum likelihood was used to determine distance among different sequences in the phylogenetic tree.

Statistical analysis

Data were analyzed by SPSS version 19.0. Descriptive statistics were used to summarize the data.

Two genera and 6 species were identified including 111 *Rhipicephalus sanguineus* (41.3%), 24 *Hyalomma detritium* (8.9%), 6 *Hyalomma marginatum* (2.2%), 9 *Hyalomma anatolicum* (3.3%), 5 *Hyalomma asiaticum* (0.9%), 90 *Hyalomma dromedarii* (33.5%) and 10 *Hyalomma spp.* (3.7%). Eleven (4.1%) *Hyalomma* nymphs and 3 (1.1%) *Rhipicephalus* nymphs were also identified. The highest frequency of genus and species was related to the *Hyalomma* and *Rhipicephalus sanguineus,* respectively. The presence of *Anaplasma* was confirmed in 20 out of 100 tested samples (20%). All positive samples collected from Birjand county were *Rhipicephalus sanguineus* (Table 1).

Out of 20 infected ticks, 5 (25%) were isolated from goats and 15(75%) from sheep. The male: female ratio of positive ticks was 12:8. Eighteen infected ticks (90%) were captured from lowland areas while two (10%) were collected from highland areas.

A phylogenetic tree was constructed between the present sequence and registered sequences of MSP-4 in GenBank. Four clades were created for *Anaplasma spp.* including: *A. ovis, A. marginale, A. capra*, and *A. phagocytophilum* (Fig1).

The predominant species in South Khorasan province was *Rhipicephalus sanguineus*. The prevalence of *Anaplasma* infection in hard ticks of the re-

Table 1.
Information related to tick species that were selected for molecular detection.

Species	No. of examined ticks by PCR	Identified specimens (No.)	Identified specimens (%)
Rhipicephalus sanguineus	61	20	32.7
Hyalomma detritium	3	0	0
Hyalomma marginatum	0	0	0
Hyalomma anatolicum	61	0	0
Hyalomma asiaticum	4	0	0
Hyalomma dromedarii	0	0	0
Hyalomma spp.	4	0	0
Total	100	20	20

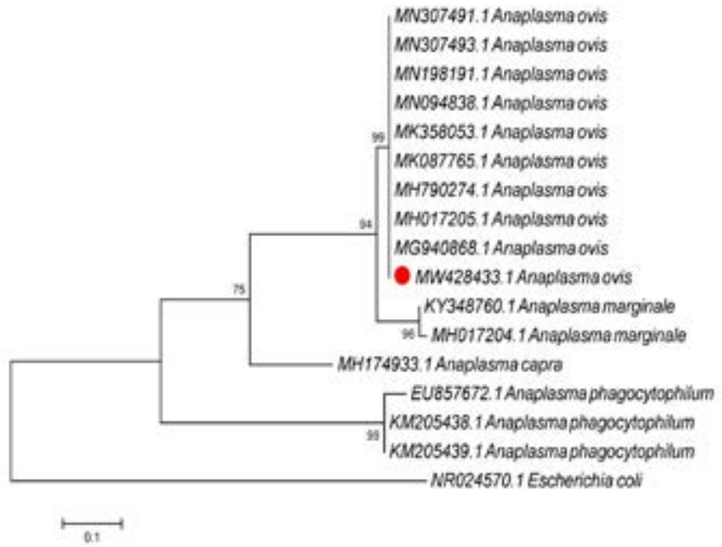

Fig 1.
The evolutionary tree was inferred by using the maximum likelihood method with bootstrap of 1000 replications. The percentage of trees in which the associated taxa clustered together is shown next to the branches. *Escherichia coli* was included as outgroup. The scale bar indicates an evolutionary distance of 0.10 nucleotides per position in the sequence [10,11]. Sequence derived from the present study is marked with a red circle.

gion was 20% which was only related to Birjand county. Infection was found in *Rhipicephalus sanguineus* as the only infected species. Infected ticks were collected from sheep and goats. Ticks collected from other livestock were negative in terms of *Anaplasma* infection. Plains were more infected than highland areas.

In a molecular survey of hard ticks in the Iran-Afghanistan borderline (Sistan region, southeast of Iran), Jafar Bekloo et al. reported that *Anaplasma* was found in 26.4% of tested specimens. The results showed the infection of *Rhipicephalus sanguineus* and *Hyalomma anatolicum* with *Anaplasma ovis* [12]. In a study conducted in the Kerman province (which shares a border with South-Khorasan) rate of tick infection with *Anaplasma* was reported at 23.95% and *Hy. Marginatum* and *Rhipicephalus sanguineus* were infected species [3]. South Khorasan, Kerman, and Sistan regions are neighbors and share common geographical attributes; so they are supposed to show a similar prevalence of tick infection associated with *Anaplasma*. Tick infection with *Anaplasma* in East-Azerbaijan province, southwest of Iran was reported 71% which is higher than east of the country [9]. Jafar Beklooa et al. also reported that tick infection with *Anaplasma* was 25% in the north of Iran and infected species were: *Rh. sanguineus*, *Rh. bursa*, *Hy. Marginatum* and *Hy. Scupense*, a finding which was very different from the present study in terms of infected tick species [13]. A gene fragment of *Anaplasma* species was identified in 49.5% and 59% of tested ticks in the Mazandaran and Savadkouh regions of Iran, respectively [1,14]. From the previous studies, it can be concluded that although the prevalence of tick infection with *Anaplasma* is variable in different parts of Iran, the most prevalent tick genus with *Anaplasma* infection is *Rhipicephalus*.

Results of the present study showed a relatively high infection rate in hard ticks collected from livestock in South-Khorasan Province. It seems that controlling and preventive policies related to the spread of anaplasmosis in South Khorasan province should be pursued more seriously. In addition, due to the common border with Afghanistan, there is a possibility of cross-border transmission. More studies in terms of tick infection with other arthropod-borne pathogens are also recommended in South-Khorasan.

Authors' Contributions

A.J., S.A., M.R. and A.H. conceived and planned the experiments. A.J., S.A., F.F. and M.F. carried out the experiments. A.J., D.S., A.L, M.B. and S.A. contributed to sample preparation. A.J., D.S., M.R., M.B. and A.L. contributed to the interpretation of the results. A.J. took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

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Competing Interests

The authors declare that there is no conflict of interest.

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